Chapter Seven

General Discussion

7.1 The role of methylation in the vernalization response

In this thesis, the hypothesis that cytosine demethylation is involved in mediating the vernalization response was tested by observing the flowering behaviour of Arabidopsis plants transformed with an antisense construct against the Arabidopsis cytosine methyltransferase *METI* (antisense methyltransferase, AMT). Plants from four independent families of AMT transgenics showed significant demethylation (Chapter 3; Finnegan *et al*., 1996). Plants from all these families flowered earlier than wildtype. The only requirement for the early flowering response was decreased methylation. The promotion of flowering in the AMT transgenics was correlated with the extent of demethylation, such that plants with more extensive demethylation flowered earlier. To test whether this early flowering response acted specifically through the vernalization pathway, the AMT transgene was crossed into two late flowering mutants which differed in vernalization responsiveness. Extensive demethylation was observed in both mutant backgrounds. The vernalization responsive *fca*-AMT line flowered significantly earlier than the *fca*-C24 control line, indicating that demethylation caused early flowering in this vernalization responsive mutant. In the *fe*-AMT line that had only a very small vernalization response, demethylation did not promote flowering relative to the control line. These results indicate that the early flowering response to demethylation operates specifically through the vernalization pathway.

A second *fe*-AMT line showed a large response to vernalization, as did one *fe*-C24 control line. These lines flowered much later than the lines showing a small vernalization response. This late flowering phenotype was not linked to the *FRI* gene, demonstrating the existence of another locus (or loci) in C24 that causes late flowering that can be overcome by vernalization. Both these lines have increased levels of *FLC* expression relative to the less vernalization responsive lines (E.J. Finnegan, personal communication), suggesting that the late flowering locus (or loci) inherited from C24 is likely to have its effect on flowering time and vernalization responsiveness by upregulating *FLC* expression.

FLC expression is up-regulated in *fca* and other vernalization-responsive late flowering mutants, and down-regulated in vernalized plants and AMT transgenics (Sheldon *et al*., 1999; Michaels and Amasino, 1999). *FLC* expression is reduced in the *fca*-AMT line relative to the control *fca*-C24 line (E.J. Finnegan, personal communication), suggesting that the promotion of flowering seen in the *fca*-AMT line is due to the effect of demethylation on the expression of *FLC*. *fe* and other late flowering mutants with a small or non-existent response to vernalization do not show increased *FLC* expression (Sheldon *et al*., 1999); this was true of the *fe*-AMT and *fe*-C24 lines that showed little vernalization response (E.J. Finnegan, personal communication). These results indicate that demethylation and cold treatment both promote flowering through down-regulation of *FLC*.

Application of gibberellic acid (GA) hastens flowering of Arabidopsis, and this hormone may play a role in the vernalization response. To examine the relationship between methylation and the role of GA in flowering, the effect of a GA-insensitive mutation in L*er*, *gai*, on flowering response to demethylation was examined. The presence of a single *gai* allele in AMT plants delayed flowering, indicating that the promotion of flowering due to demethylation requires a functional GA signal transduction pathway.

The promotive effect of demethylation by *METI* antisense does not completely substitute for cold treatment, indicating that the vernalization process involves components other than *METI*-mediated methylation. It is possible that cytosine methyltransferases other than *METI* are involved in the vernalization response. Finnegan and Dennis (1993) found that Arabidopsis contains a small family of cytosine methyltransferases, and one of these, *METIIa*, was characterised.

METI and *METIIa* are extremely similar in structure and sequence. *METI* is likely to be a functional methyltransferase, as *METI* antisense plants have decreased methylation (Finnegan *et al*., 1996). The similarity of *METIIa* to *METI* and other eukaryote methyltransferases suggests that it is also likely to encode a functional methyltransferase. Both *METI* and *METIIa* are expressed in all tissues and developmental stages assayed, though *METI* is expressed at a much higher level than *METII*. A third gene, *METIIb*, is sufficiently similar to *METIIa* that the expression ascribed to *METIIa* could originate from either gene (Genger *et al*., 1999).

7.2 Future work

7.2.1 Down-regulation of *FLC* by vernalization and demethylation

Down-regulation of *FLC* is central to the vernalization response, and is mediated by demethylation (Sheldon *et al*., 1999). The expression of *FLC* could be directly affected by demethylation, or down-regulation of *FLC* could occur due to demethylation and consequent up-regulation of a *FLC* regulatory gene. The number of steps between a putative gene regulated by changes in methylation, and a gene product that regulates *FLC* expression, is not known. Two approaches can be taken to investigate the regulation of *FLC* by vernalization: examining the role of methylation in affecting the expression of *FLC*, and genes that directly or indirectly regulate *FLC*; and searching for gene products that directly affect *FLC* expression. These approaches are outlined below.

To test whether *FLC* expression is regulated by changes in cytosine methylation after vernalization, methylation within the *FLC* gene can be compared between unvernalized and vernalized plants, and between wildtype and AMT plants. The promoter region, introns and coding regions of the gene should be studied. The bisulphite sequencing technique (Frommer *et al.*, 1992; Clark *et al*., 1994) is being used to make these comparisons (E.J. Finnegan, personal communication). Plants carrying reporter genes fused to the *FLC* promoter, or to the promoter and other *FLC* regions, have been generated, and the expression of the reporter genes after vernalization is being studied

(C.C. Sheldon, personal communication); this will allow identification of regions of *FLC* that increase *FLC* expression in response to vernalization or demethylation.

If changes in cytosine methylation within *FLC* can be correlated with changes in expression after vernalization, cytosines showing the changes could be mutagenized, and the mutagenized gene transferred into a null mutant for *FLC*, so that the effect on expression in response to vernalization could be observed. If vernalization does not lead to a change in the expression of the mutagenized transgene, this will suggest that the expression of *FLC* is directly regulated by cold-induced demethylation. If no changes in methylation within *FLC* are seen after vernalization, or if such changes do not affect *FLC* expression, another gene that regulates *FLC* expression may be affected by cold-induced demethylation. Candidate genes that may be regulated by methylation, and/or may directly regulate *FLC* expression, are discussed below.

FLC is down-regulated by genes that act in the autonomous pathway, including *FCA*, *FY*, *FVE*, *FPA*, and *LD* (Sheldon *et al*., 1999; Michaels and Amasino, 1999). The order in which these genes act is not certain; they may form a signalling cascade, in which one or more genes acting late in the cascade down-regulate *FLC* directly or indirectly. The *VRN2* gene also down-regulates *FLC* independently of the vernalization response, as the *fca vrn2* mutant flowers later and has higher *FLC* expression than *fca* (Chandler *et al*., 1996; Sheldon *et al*., 1999). As well as this independent role, *VRN2* and also *VRN1* down-regulate *FLC* through the vernalization pathway, as mutations in either *VRN* gene decrease the vernalization response and the decrease in *FLC* expression seen after

vernalization (Chandler *et al*., 1996; Sheldon *et al*., in press). However, the *vrn1* mutation does not cause elevation of *FLC* expression, indicating that *VRN1* affects *FLC* only through the vernalization response (Sheldon *et al*., in press). The *vrn1* mutation does not delay flowering of *fca*, but does delay flowering when segregated away from *fca* (Chandler *et al*., 1996), suggesting that *VRN1* promotes flowering by a pathway independent of *FLC*, and acts downstream of *FCA* (Figure 7.1a).

The down-regulatory effects of demethylation, *VRN2* and the Class 1 genes may act on *FLC* independently, so that the vernalization and autonomous pathways converge at *FLC* (Figure 7.1a). Alternatively, the pathways may converge upstream of *FLC*, and affect the expression of a gene that regulates *FLC*. This could be a gene such as *VRN2*, which down-regulates *FLC* (Figure 7.1b).

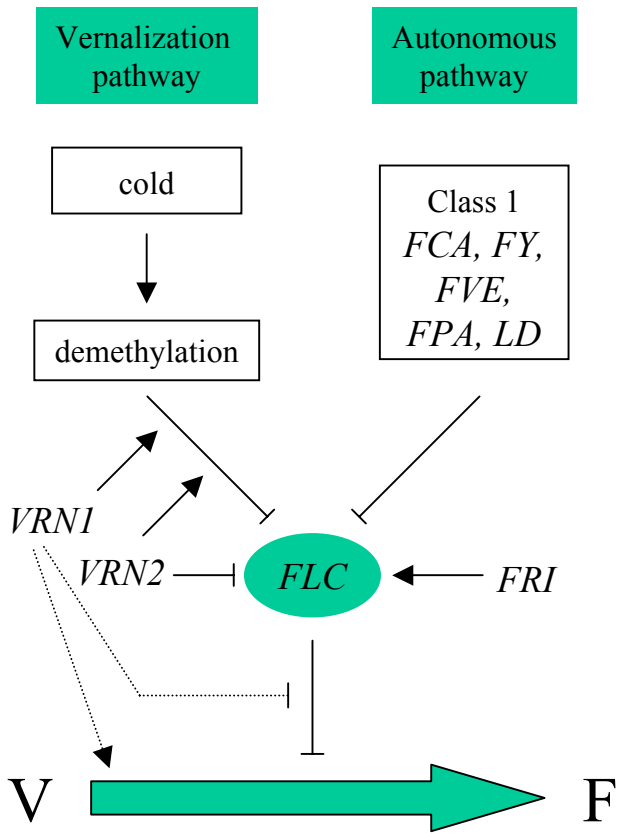
VRN2 is a strong candidate to mediate the down-regulation of *FLC* (Figure 7.1b) as plants carrying *vrn2* mutations show increased *FLC* expression and delayed flowering. *fca-1 vrn2* double mutants flower later and have higher *FLC* expression than *fca*, suggesting that *vrn2* may also have a role in down-regulating *FLC* that is separate to its putative role linking the vernalization and autonomous pathways. However, neither the *fca-1* nor *vrn2* are definitely known to be null alleles; if these mutants are leaky, the *fca-1 vrn2* phenotype is consistent with the hypothesis that these genes could act in the same pathway (Figure 7.1b). Once the *VRN2* gene is cloned, its expression level in the Class 1 mutants can be determined, which will indicate whether these genes up-regulate *VRN2*, as suggested in Figure 7.1b. Additionally, if the *vrn2* mutation can be segregated away from

Figure 7.1: Alternative models of pathways to flowering, focusing on the mode of regulation of *FLC* through the the vernalization and autonomous pathways. Genes which are believed to act in the same pathway are grouped in boxes. Promotive effects are shown by "→", while repressive effects are shown by "⊣". Alternative pathways are shown by dotted lines.

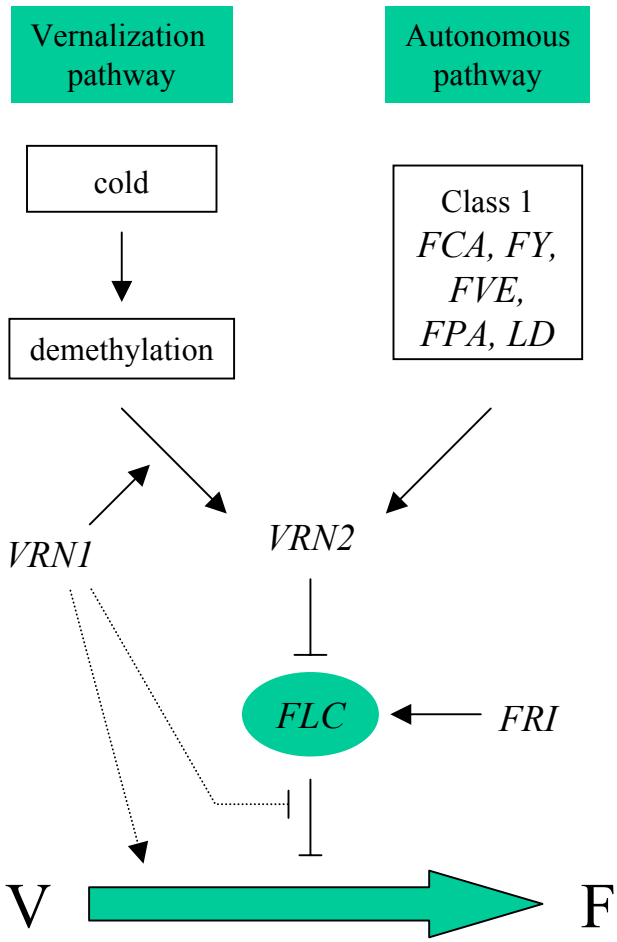
(A) Independent down-regulation of *FLC* by demethylation, Class 1 genes and *VRN2*.

(B) direct down-regulation of *FLC* by *VRN2*, resulting from up-regulation of *VRN2* by demethylation and by the Class1 genes.

(A)


Vernalization pathway
Autonomous pathway
cold
demethylation
Class 1
FCA, FY,
FVE,
FPA, LD
VRN1
VRN2
FLC
FRI
V
F

(B)


Vernalization pathway
Autonomous pathway
cold
demethylation
Class 1
FCA, FY,
FVE,
FPA, LD
VRN1
VRN2
FLC
FRI
V
F

fca, it can be crossed to other Class 1 mutants. If *VRN2* acts downstream of the Class 1 genes, the phenotype of these double mutants should be additive, or the Class 1 mutations should be epistatic to *vrn2*, depending on the leakiness of the mutants.

It is possible that one of the Class 1 genes, rather than *VRN2*, could act as the link between the vernalization and autonomous pathways. This appears less likely due to the vernalization response shown by mutants for these genes. It is possible, however, that the mutant allele of one of these genes retains a partial down-regulatory effect on *FLC*, and that vernalization increases expression of the mutant allele enough to cause down-regulation of *FLC* and promote flowering. The effect of vernalization on expression is not known for any of these genes, and at present only *FCA* and *LD* have been cloned. Neither of these two genes is likely to fill the proposed role, as for both loci, mutants thought to be null (*fca-1* and *ld-3*) are strongly responsive to vernalization (Koornneef *et al*., 1991; Lee *et al*., 1994b; Macknight *et al*., 1997). Once other genes of this class are isolated, the effect of vernalization on their expression can be determined, as can the leakiness of the mutant alleles.

Further studies are needed on the *VRN2* and Class 1 genes to determine the mechanism of *FLC* down-regulation. To determine if any of these genes are regulated by methylation, the expression levels and methylation patterns can be compared between unvernalized and vernalized plants, and wildtype and AMT plants. Regions of the genes that mediate expression changes due to vernalization or demethylation could be identified by fusing the promoter, plus or minus other regions, to a reporter gene, and observing reporter gene

expression in transgenic plants after vernalization or demethylation. If genes showing a correlation between demethylation and expression are identified, further work will be needed to confirm the role of methylation in regulating expression of this gene(s). As described for *FLC*, cytosines for which changes in methylation are observed could be mutagenized to prevent methylation at those sites. Constructs carrying the mutagenized gene could be transformed into null mutants for that gene, so that the effect on expression and the flowering time response to vernalization could be observed. If vernalization and AMT-induced demethylation have no effect on the expression of the mutagenized gene or on flowering time, it is likely that changes in methylation pattern are required for up-regulation of the gene and for promotion of flowering.

Analysis of the *FLC* promoter will also provide information on the mechanism of *FLC* down-regulation. The protein products of genes that are candidates for direct down-regulators of *FLC* can be used in gel shift assays with the *FLC* promoter to determine whether they bind to it. Gene products that bound to the promoter could be tested to determine whether they inhibited transcription of *FLC in vitro*. Gel shift assays could be used for deletion analysis of the *FLC* promoter, to determine the region where binding occurs. In addition, transgenic plants carrying a series of promoter-GUS fusion constructs with deletions of different promoter regions could provide information on the region of the promoter required for down-regulation after vernalization or demethylation.

7.2.2 The role of GA in the vernalization response

The preliminary results from the experiments with *gai* reported in Chapter Five suggest that the GA signal transduction pathway operates downstream of the methylation-regulated step in the vernalization pathway; studies into the role of *FLC* suggest that *FLC* is regulated by methylation and negatively regulates GA responses (Sheldon *et al*., 1999). *FLC* expression is not affected by exogenous GA (Sheldon *et al*., 1999). The implications of these findings justify a more thorough examination of the relationship between methylation, *FLC* and GA.

The experiments with the *gai* mutant reported in Chapter Five used F1 plants from crosses between *gai* and C24, *gai* and AMT, or L*er* and AMT. As the *gai* mutation is semi-dominant (Peng and Harberd, 1993; Wilson and Somerville, 1995), the GA responsiveness of the *gai*-C24 or *gai*-AMT F1 plants is likely to have been greater than that of homozygous *gai* mutants. Further experiments on F1 plants from crosses with *gai* should include tests of the GA responsiveness of the *gai*-C24 or *gai*-AMT F1 plants relative to control F1 plants, from L*er*-C24 and L*er*-AMT crosses.

The problem of altered GA responsiveness in experimental plants could be avoided if the AMT transgene was transformed into *gai* directly, rather than through crossing. However, the background ecotype of *gai*, L*er*, has a low level of *FLC* expression (Sheldon *et al*., 1999); as methylation appears to affect flowering through regulation of *FLC*, it may be necessary to transform a *gai* mutant containing an allele of *FLC* that is

expressed at high levels, such as the alleles from ecotypes Pitztal or San Feliu-2 (Sheldon *et al*., 1999). If, as suggested by the preliminary experiments reported here, GA signal transduction is required for the early flowering response to demethylation, the flowering time of *gai* homozygotes with AMT-induced demethylation should not be promoted.

Another approach to determine the relationship of *FLC* and GA responses would be to screen for mutants with a lesion downstream of *FLC* in the pathway to flowering. Such mutants could be identified by mutagenizing a vernalization responsive ecotype, such as C24, that expresses *FLC* at high levels, and screening for mutants that flower early and do not respond to vernalization despite expressing *FLC* at wildtype levels. As *FLC* appears to negatively regulate GA responses, and as GA signal transduction appears to be essential for the early flowering response to demethylation, these mutants could be affected in GA signal transduction. This could be determined by examining flowering and phenotypic responses to exogenous GA, and by crossing to mutants such as *gai* to determine allelic or epistatic relationships.

In *Thlaspi*, a close relative of Arabidopsis, vernalization increases the activity of kaurenoic acid hydroxylase (KAH), an enzyme that catalyzes an early step in GA biosynthesis (Hazebroek and Metzger,1990; Hazebroek *et al*., 1993). This observation suggests that *FLC* may down-regulate KAH activity. To investigate this possibility, the effect of over- and under-expression of *FLC* on endogenous GA levels and on the levels of GA intermediates could be measured. Such studies would be best done in *Thlaspi*, as

changes in GA metabolism affecting flowering time may occur only in the shoot apex, and the larger size of the *Thlaspi* apex would be an advantage.

7.2.3 Other Arabidopsis cytosine methyltransferases

Five potentially functional cytosine methyltransferases have been identified in Arabidopsis, and more may yet be found (Finnegan and Dennis, 1993; Genger *et al*., 1999; Henikoff and Comai, 1999). Possible rationales for the presence of multiple methyltransferases in Arabidopsis include functions in maintenance versus *de novo* methylation, methylation of cytosines in different sequence contexts, and activity only in specific tissues or developmental stages. The three methyltransferases of the METI family are most likely to function in maintenance methylation, as all possess a large amino terminal domain with similarity to the Dnmt1 amino terminal domain, which confers a preference for hemi-methylated DNA on that enzyme (Bestor, 1992). The sequence divergence between the *CMT* genes and the *METI*-like genes, as well as the presence of a chromodomain in the CMT methyltransferases, suggests that these classes are likely to fulfil different roles. Chromodomains target proteins to heterochromatin (Paro and Harte, 1996); CMTI and CMTII may methylate cytosines within heterochromatin, or participate in the process of condensation of DNA into heterochromatin.

Further work is required to determine the roles of the Arabidopsis cytosine methyltransferases so far identified. Analysis of the phenotypes and changes in cytosine

methylation seen in antisense transgenics or knockout mutants can provide important information. The demethylation observed in *METI* antisense transgenics indicates that METI targets cytosines in CG and CCG sequences. So far, no phenotype or demethylation has been observed for *METIIa* antisense transgenics (K.A. Kovac, personal communication), in which *METIIb* expression may also be disrupted. The effectiveness of the antisense transgene can be determined by RT-PCR assays for the *METII* genes. If the expression of these genes is shown to be disrupted in the antisense transgenics, they may be non-essential for normal development. Transgenics carrying antisense constructs for both the *METI* and *METIIa* genes can be studied to determine whether the *METI* antisense phenotype is exacerbated by *METIIa* antisense, and particularly whether the promotion of flowering by *METI* antisense-mediated demethylation is increased, or whether the resetting mechanism for the vernalization response, which is intact in *METI* antisense plants (Finnegan *et al*., 1998), is disrupted. No antisense transgenics or knockout mutants have yet been generated for the *CMT* genes, although four Arabidopsis ecotypes produce only a truncated version of the CMT1 protein, indicating that it is not essential for normal development (Henikoff and Comai, 1998). *CMT1* function may be redundant to another *CMT* methyltransferase, in which case it may be necessary to disrupt more than one *CMT* gene in order to produce an abnormal phenotype or change methylation patterns.

So far, analyses of the expression patterns of genes in the *MET* and *CMT* classes have involved fairly crude bulks of tissue types and limited sampling of developmental stages. If the *METII* genes, like *CMTI*, prove to be non-essential genes, any phenotype caused by

disruption of their activity is likely to be subtle. More detailed analysis of the expression patterns of these genes, as well as of essential genes like *METI*, will provide more information on the cell types and stages in which they are active, and in turn this may indicate possible phenotypes which may be apparent on closer investigation. *In situ* analyses, possibly using promoter-GUS fusions for ease of detection, would provide a more detailed picture of the expression patterns of these methyltransferase genes.

For all these genes, *in vitro* assays of protein function will be required to define their target sequences. Cytosine methyltransferase enzymes have been isolated from wheat and pea and shown to have methyltransferase activity *in vitro* (Theiss *et al*., 1987; Pradhan and Adams, 1995). This approach is probably not viable for Arabidopsis, for the following reasons. *METI* is the most highly expressed Arabidopsis methyltransferase, but is expressed at lower levels than the pea methyltransferase, which can be readily detected by Northern analysis (Pradhan *et al*., 1998). To extract sufficient methyltransferase for *in vitro* assays, Pradhan and Adams started with 4-5 kg of pea apices; this is impractical for Arabidopsis apices. For the *METII* and *CMT* genes, which are expressed at much lower levels than *METI*, it would be practically impossible to extract sufficient enzyme. Pradhan *et al*. (1998) reported baculovirus-mediated expression of the pea cytosine methyltransferase cDNA; this method may be successful for the Arabidopsis enzymes also. *In vitro* assays will allow preferences for hemi-methylated or unmethylated substrates, and for cytosines in different sequence contexts, to be determined. Also, these assays will give an indication of the relative activities of the native proteins, though

differences between plants and bacteria in post-translational modification and protein folding may cause bias.

7.3 Final conclusions

The importance of cytosine methylation in plants can no longer be ignored, as evidence accumulates for the role of cytosine methylation in processes as diverse as genome defence, development, epigenetic processes and flowering. The work reported here, interpreted in the light of the current literature, demonstrates that cytosine methylation has a significant role in regulating the timing of flowering through the vernalization response. Essential steps in understanding the mechanisms by which methylation affects processes such as flowering can be taken, now that genes likely to be regulated by methylation, such as *FLC*, have been cloned. The identification of multiple cytosine methyltransferase genes, and the continuing efforts to understand the functions of these genes, will also allow major advances in understanding how plants establish, maintain, and modify patterns of cytosine methylation.